METHODS AND COMPOSITIONS FOR COMBINATION RNAi THERAPEUTICS

This application claims priority to U.S. Provisional application Serial No. 60/541,776, filed February 5, 2004, the contents of which are hereby incorporated by reference in their entirety.

Field of Invention

The present invention provides methods and compositions for using a combination of RNAi agents. Such methods include treatments for cancer, infectious diseases and inflammations. The combined RNAi agents of the invention may comprise separate species, *i.e.* in "trans", or single species, *i.e.* in "cis". In one embodiment, the combination is an siRNA oligoucleotide cocktail (siRNA-OC) that contains multiple short dsRNA duplexes targeting multiple drug targets.

The present invention is based on two important recognitions: (1) RNAi comprises very potent nucleic acid therapeutic agents comprised of sequences (21-23 nt, or 24-25 nt, or 26-29 nt) matching a segment of a targeted drug but otherwise having the same chemistry properties; and (2) most disease is controlled by multiple biochemical pathways usually controlled by multiple genes and proteins, and often times a combination of endogenous and exogenous factors. Therefore, using a combination of RNAi agents targeting multiple disease controlling genes represents an advantageous therapeutic approach, due to the chemical uniformity of siRNA duplexes and synergistic effect from down regulation of multiple disease causing genes. According to the present invention, siRNA-OC is a combination of siRNA sequences targeting multiple genes, in some instances at various proportions, in some instances with multiple physical forms (e.g. solution or powder), and in some instances being applied through the same route at the same time and in other instances through different routes or different times.

Background

Human disease is a complicated pathological process manifested in varying severities of disease symptoms. Many human diseases are caused by abnormal over expressions of disease causing or disease controlling genes from the human body itself, or from foreign infectious organisms, or both. Disease progression and development of drug resistance can circumvent the effect of single drug treatment. One strategy to overcome these hurdles is to use a combination of multiple drugs.

The combination of drugs is standard practice in medicine, most often managed by each patient's physician. This is especially clear in oncology where combinations of chemotherapies developed for each class of cancer have achieved remarkable improvements in anti-cancer efficacy and have reduced toxicities. One example is the use of docetaxel, ifosfamide and cisplatin combination therapy for treatment of oropharyngeal cancer with multiple bone metastases from prostate cancer (2). Another example is the treatment of ulcerative colitis with a combination of corticosteroids, Metronidazole and Vancomycin (3). Yet another example is the treatment of insufficiently controlled type 2 diabetes by adding rosiglitazone to a combination of glimepiride and metformin therapy (4). Although these clinical studies have demonstrated remarkable therapeutic benefits, the potential for toxicities and adverse interactions is always a major concern, due to the fact that different drugs have different chemical properties, pharmacokinetics, pharmacodynamics, etc. Hence, deriving the benefit from combination of drugs has been relegated to post-marketing with oversight by practicing physicians. Recently, however, the combination of retroviral agents in a single product has achieved clinical benefits for treatment of AIDS. This combination still depends on a mixture of drugs with different chemical and pharmacological properties, which complicates development and continues to pose risk for patients. Therefore, a need exists for a means to control the multiple aspects of disease, wherein such means is based upon work with an animal model of the disease prior to clinical studies or prior to post-marketing stages of drug use. This would reduce the risk of adverse interactions due to differences in pharmacology (e.g. pharmacokinetics and pharmacodynamics) associated with the different chemistries of the drugs used in the combination. This need could be met by cocktails of drugs, wherein the drugs have uniform pharmacology.

Summary of The Invention

It is therefore an object of the invention to provide compositions that are useful, for example, for treating disease, where the compositions contain combinations of siRNA duplexes that inhibit gene expression.

It is a further object of the invention to provide improved methods of treating disease using combinations of siRNA duplexes that inhibit gene expression.

In accordance with these objects there is provided a composition containing at least two siRNA duplexes and a pharmaceutically effective carrier where each of the siRNA duplexes inhibits expression of a gene associated with a disease process. The genes may be distinct genes.

In one embodiment the composition comprises at least three siRNA duplexes and the siRNA duplexes may, for example, inhibit expression of at least three gene sequences including three open reading frames and three mRNAs. The genes may be endogenous human genes and/or they may be exogenous genes of one or more pathogens.

The compositions may be used to treat a disease is selected from the group consisting of cancer, autoimmune and inflammatory diseases, and other diseases caused or exacerbated by abnormal over expression of multiple genes.

In another embodiment the siRNA duplexes may be chemically synthetic forms, containing naturally occurring RNA bases and/or chemically modified RNA bases of RNA base analogs.

The composition may be administered, for example, via local injection, inhalation, topical cream, dermal patch, or systemic delivery by IV, IP or IM injection.

In one embodiment, the composition may contain aagctcctaattacactcaac; aaggatgaggaaggcaattta; aaggataagtcagctcaatgc; and aactggcacactacttgtcga, and may be used, for example, for treating a coronavirus in a subject

In another embodiment, the composition may contain AAGCCGTCCTGTGTGCCGCTG; AACGATGAAGCCCTGGAGTGC; AAGTTAAAAGTGCCTGAACTG; AAGCAGGCCAGACTCTCTTTC; AAGCTCAGCACACAGAAAGAC; and AATGCGGCGGTGGTGACAGTA, and may be used, for example, to treat ocular disease.

In still another embodiment, the composition may contain a combination of siRNA duplexes that inhibit expression of VEGF, VEGF R2 and VEGF R1, where the duplexes are selected from Figure 17, and the disease may, for example, be selected from

the group consisting of ocular neovascularization diseases such as wet AMD, diabetic retinopathy and stromal keratitis, various types of cancers, rheumatoid arthritis and lung angiogenesis disease.

In yet another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of VEGF, VEGF R1 and VEGF R2 and are selected from Figure 17 SS1, SS2 and SS3, and may be used, for example, to treat cancer, ocular neovascularization, such as wet AMD, diabetic retinopathy, peripheral retinal neovascularization, or glaucoma.

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of EGF receptor, VEGF and FGF, and that are selected from Figure 17 SS1, SS2 and SS3. In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of EGF receptor, VEGF receptor and FGF receptor, and are selected from Figure 17 SS1, SS2 and SS3. These compositions may be used, for example, in a method of treating cancer in a subject.

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of androgen receptor, VEGF and AMACR, and are selected from Figure 17 SS2 and SS53, and the disease may be prostate cancer.

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of VEGF, c-Met and PCDP10, and are selected from Figure 17 SS2, SS4 and SS5, and the disease may be liver, lung or colon cancer.

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of HGF, c-Met and VEGF, and are selected from Figure 17 SS2, SS3, and the disease may be liver cancer.

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of EGF receptor, VEGF and p53 mutants, and are selected from Figure 17 SS1, SS2 and SS3, and the disease may be lung cancer.

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of HPV16 and HPV18's E6, E7 and human p53 mutants, and the disease may be cervical cancer.

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of MMP-2, PDGF-R and $\alpha v\beta 3$ integrin, and the disease may be cancer

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of TNF alpha, IL-1 and IL-1 receptor, and the disease is an an inflammatory disease, for example, rheumatoid arthritis, uveitis, psoriasis or Crohn's disease.

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of IL-9, IL-4 and IL-5, and the disease may be asthma, pulmonary fibrosis or ARDS.

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of viral nucleocapsid protein, nonglycosylated inner virion protein and a transmembrane glycoprotein (F) of RSV virus, and that are selected from Figure 17, SS6, and the disease may be an RSV infection.

In another embodiment, the composition may contain a combination of siRNA duplexes that target mRNA sequences of Spike protein, RNA polymerase and RNA replicase of SARS-CoV, and that are selected from Figure 17 SS7, and the disease may be SARS.

In a further embodiment there is provided a method of target validation by administering a composition as described above to a test subject and measuring changes in gene expression in the subject.

Description of the Figures

Figure 1. siRNA-mediated *in vitro* knockdown of VEGF pathway genes. RAW264.7 NO (-) cells (**A**) and SVR cells (**B**) in 35-mm wells were transfected with siRNA (equimolar mixture of two targeted sequences a + b) targeting mVEGFA and mVEGFR1, respectively, at the amount indicated. The 293 cells (**C**) were co-transfected with siRNA targeting mVEGFR2 (equimolar mixture of two targeted sequences a + b was used in subsequent experiments) and plasmid expressing mVEGFR2 at the amount indicated. Cellular RNA was isolated 24 hours (RT-PCR) or 48 hours (RS-PCR) after transfection, and the knockdown of endogenous expression of mVEGFA or mVEGFR1, or exogenous expression of mVEGFR2 was measured by RT-PCR for mVEGFA, or RS-PCR for mVEGFR1 and mVEGFR2.

Figure 2. Local delivery of siRNAs targeting VEGF pathway genes inhibits the CpG ODN-induced angiogenesis. Twenty-four hours after implantation with CpG ODN (1 _g) into the micropocket in mouse cornea, the mouse was given (10 ug per eye) siLacZ,

siVEGFA, siVEGFR1, siVEGFR2, or siVEGF mix (an equimolar mixture of total siRNAs targeting VEGF pathway genes) by subconjunctival injection. The angiogenesis area was measured on days 4 and 7 after the CpG pellet implantation (four mice per group). **A:** Statistically significant differences in angiogenic areas (*, $P < 0.05$; **, $P < 0.01$) were observed between the groups. **B:** Images were taken by stereomicroscopic imaging system at day 7 after CpG pellet implantation. Original magnifications, x40.

Figure 3. Systemic delivery of siRNAs against VEGF pathway genes inhibits the CpG ODN-induced angiogenesis. Individual siRNAs or a mixture of total siRNAs against VEGF pathway genes were delivered with TargeTran, 6 and 24 hours after the CpG ODN induction by tail vein injection. The angiogenesis area was measured on days 4 and 7 after the CpG pellet implantation (four mice per group). **A:** Statistically significant differences in angiogenic areas (*, $P < 0.05$; **, $P < 0.01$) were observed between the groups. **B:** Images were taken by stereomicroscopic imaging system at day 7 after CpG pellet implantation. Original magnifications, x40.

Figure 4. Increased efficiency of siRNA systemic delivery by polymer and dose-response experiment. A mixture of siVEGF pathway genes or siLuc control (40 ug) with polymer or PBS control was delivered 6 and 24 hours after the CpG ODN induction. The angiogenesis area was measured on days 4 and 7 after the CpG pellet implantation (four mice per group). **A:** Statistically significant differences in angiogenic areas (*, $P < 0.05$) were observed between the groups. Dose-response (10, 20, 40, and 80 ug siRNA) experiment with mixed siRNAs targeting VEGF pathway genes with polymer in the systemic delivery system was performed. **B:** The angiogenesis area in each of the four mice for each group was measured on days 4 and 7 after implantation of CpG ODN, and the anti-angiogenic efficiency was compared between different siRNA dosages.

Figure 5. Reduced HSK severity and angiogenic response by administration of siRNAs targeting VEGF pathway genes. Mice were infected with 1 x 10 exp5 PFU HSV-1 RE per eye, and at days 1 and 3 after infection, were treated with siVEGF mix or siLuc with polymer either locally or systemically. Mean lesion HSK score (**A**) and angiogenic score (**B**) were calculated at day 10 after viral infection. Each **dot** represents the clinical score for one eye. **Horizontal bars** and figures in the **parentheses** indicate the mean for each group. Data are complied from two separate experiments consisting of six eyes in each group. *, Statistically significant differences in HSK or angiogenesis score ($P < 0.05$) were observed between the groups. At day 14 after infection extensive growth of blood

vessels and ulceration were seen in the infected cornea of siLuc-treated mice. **C:** siVEGF mix-treated mice showed less neovascularization near the limbal area.

Figure 6. Decreased level of VEGF mRNA in the cornea that was infected and treated with siVEGF mix with either local or systemic delivery. Two corneas were collected at days 4 or 7 after infection from mice that were infected with 1 x 10 exp5 PFU HSV-1 RE and were treated with siRNAs targeting VEGF pathway genes at days 1 and 3 after infection by either local (10 ug, subconjunctival; S/C) or systemic (40 ug, tail vein) administration and then VEGF mRNA level was measured by RT-PCR (**A**) or quantitative real-time PCR (**B**).

Figure 7. Reduced levels of VEGF protein in the cornea that was infected and treated with siVEGF mix with either local or systemic delivery. At day 7 after infection two corneas per mouse were processed to measure the VEGF protein levels. Levels of VEGF were estimated from supernatants of corneal lysates of mice infected with HSV-1 and treated with siRNAs targeting VEGF pathway genes by an antibody capture ELISA as outlined in Materials and Methods. Results are expressed as mean SD of four separate mice (two corneas per mouse). *, Statistically significant differences in VEGF protein levels ($P < 0.05$) were observed between the groups.

Figure 8. SARS coronavirus yield changes after the host cells were infected by the virus and then treated with siRNA duplexes, SC2, SC5, SC14 and SC15.

Figure 9. Combination of siRNA inhibitors resulted in much improved SARS coronavirus inhibitor compared to the single siRNA inhibitor, and even to the increase the dosage, measured by the viral genome copies with RT-PCR.

Figure 10. Combination of siRNA inhibitors resulted in much improved SARS coronavirus inhibitor compared to the single siRNA inhibitor, and even to the increase the dosage, measured by the viral titers with plaque assay.

Figure 11. siRNA-mediated inhibition of tumor growth. Using ICT-1053 specific siRNA delivered by intratumoral administration to knockdown PDCD10 expression, resulted in tumor growth inhibition.

Figure 12. siRNA-mediated inhibition of tumor growth. Using ICT-1052 specific siRNA delivered by intratumoral administration to knockdown c-Met, resulted in tumor growth inhibition.

Figure 13. siRNA-mediated inhibition of tumor growth. Using ICT-1027 specific siRNA delivered by intratumoral administration to knockdown Grb2 gene expression, resulted in tumor growth inhibition.

Figure 14. siRNA knockdown of ICT1052 and ICT-1053 genes in the cell culture (MDA-MB-435 cell) study resulted in significant inhibition of cell proliferation activity.

Figure 15. siRNA-mediated activation of apoptosis in tumor cells. Using ICT-1027 specific siRNA to knockdown Grb2 gene expression, resulted in activation of apoptosis 48 hours post transfection.

Figure 16. Combined siRNA inhibitors (SC2 and SC5) suppressed SARS symptoms in Macaque Model. (a) The average temperatures of each group at each day are illustrated as: (IC) by open circles, (NS) by open squares, (PL) by closed diamonds, (CD) by closed squares and (PE) by closed triangles. The regression analysis was calculated from the raw data of each group as marked in the figure. The average body temperature of each group was calculated with 4 individuals before 7 d.p.i. and 2 individuals afterward. (b) Anti-SCV antibody was detected from the serum samples collected on 10 d.p.i., from the control groups and PL group. The titers increased until 19 d.p.i. when the samples from CD and PE groups were also turning to positive. (c) The average lung histopathological scores of each control and treatment groups have been compared. (*) indicates $P<0.05$. (D) The quantitative comparison of the SCV infected cell counts among groups. (*) indicates $P<0.05$

Figure 17 (Appendix II) shows siRNA targeting sequences for use in combination therapies.

Detailed Description of the Invention

The invention provides for combination of RNAi agents, either as multiple molecular entities or as a single molecular entity, for the treatment of disease. Most diseases are controlled by multiple biochemical pathways and most biochemical pathways are controlled by multiple factors, usually genes and proteins. The invention provides for RNAi combinations effective for these diseases, comprising RNAi effective for multiple biochemical pathways or multiple factors of a single biochemical pathway or both. In one embodiment, the multiple biochemical pathways are endogenous mammalian pathways and factors; in another embodiment, the biochemical pathways and factors span mammalian and infectious viral pathways; and in yet another embodiment, the

biochemical pathways and factors are entirely of infectious virus. The methods of the invention can be used to treat a variety of diseases and involved with a variety of pathways, as discussed below.

Cancer:

Cancer is the disease caused by multiple genetic factors and environmental hazards. The inherent oncogenes and mutation of protooncogenes are predominant contributors to various types of the cancers. Many of these genes are very well characterized: K-ras, c-Myc, a-raf and Bcl-2, etc. Over expression of various growth factors, FGF, VEGF, PDGF, EGF, and mutant tumor suppressor, Rb and p53, is the typical character of malignant tissues. Cancer or pre-cancerous growth is frequently a consequence of proliferative cellular pathologies and generally refers to malignant tumors. Malignant tumors penetrate and destroy local tissues. Some malignant tumors spread throughout the body via blood or the lymphatic system, and their unpredictable and uncontrolled growth makes malignant cancers dangerous, and fatal in many cases. Such tumors are not morphologically typical of the original tissue and are not encapsulated. Malignant tumors commonly recur after surgical removal. Accordingly, treatment of proliferative diseases ordinarily targets proliferative cellular activities such as occur in malignant cancers or malignant tumors with a goal to intervene in the proliferative processes. Certain cellular biochemical pathways are activated at different stages of the proliferative processes.

Hypoxia (inadequate oxygen), one of the key early initiators of angiogenesis, is followed by production of nitric oxide synthetases responsible for governing vascular tone and regulating growth factors such as vascular endothelial growth factor (VEGF), angiopoietins, fibroblast growth factors, and their receptors. Genes involved in matrix metabolism, including matrix metalloproteinases, plasminogene activator receptors and inhibitors, and collagen prolyl hydroxylase have also been reported as critical players in the angiogenesis process. Functional validation of specific angiogenic factors for their specific role has been greatly facilitated by use of RNAi inhibitors revealing a network involving early activation of the VEGF pathway, interactions among matrix metalloproteases and adhesion molecules, leading to regulation of signal transduction pathways. The importance of tumor angiogenesis has been widely accepted for its role in the growth and development of solid tumors (5). It is now recognized that angiogenesis is

not only essential for tumor growth, but is also implicated in the initial progression from a pre-malignant tumor to an invasive cancer, and in the growth of dormant micro metastases into clinically detectable metastatic lesions.

Factors in VEGF pathway

The VEGF family consists of five members that bind to and activate three distinct receptors. VEGF-A binds to VEGFR1 and VEGFR2, and placental growth factor (PIGF) and VEGF-B bind only to VEGFR1. VEGF-C and VEGF-D bind to VEGFR2 and VEGFR3. A number of disease-related processes function to up-regulate VEGF expression. Transcription factor HIF-1 is a key determinant of hypoxia regulated gene expression. Inhibition of HIF-1α using specific siRNA markedly attenuated the induction of expression of heme oxygenase I (HO-1), phosphoglycerate kinase (PGK), and VEGF (6), indicating a role of HIF-1α in oxygen-dependent cell cycle regulation. The role of progesterone receptor (PR) B in preferentially regulation of VEGF expression in breast cancer cells was identified using the specific siRNA (7). Through cell based assays, expression of $VEGF_{165}$ was specifically knocked down using siRNA duplexes in HeLa cells, ovarian carcinoma cells and melanoma cells (8). In a different study, the specific siRNA duplexes were able to silence an inhibitory splice variant of VEGF ($VEGF_{165b}$), although the anti-VEGF antibodies can not distinguish the differences of this variant from other isoforms of $VEGF_{165}$ (9). Using these specific siRNAs, the function of this inhibitory splice variant was further characterized that even high VEGF production in the glomerulus did not lead to angiogenesis. The existence of this splicing switch of VEGF expression represents an interesting point of intervention for cancer therapy using siRNA inhibitors. Inhibition of breast tumor growth by intratumoral delivery of siRNA duplexes targeting VEGF has been demonstrated with MDA-435 xenograft model (10). Using atelocollagen as a delivery carrier for VEGF-siRNA intratumoral administration, suppression of tumor angiogenesis and growth was also observed in a PC-3 xenograft model (11). For a different disease indication, VEGF-siRNA was used as an inhibitor of choroidal neovascularization (CNV) induced by laser photocoagulation in a murine retina model (12). Using a ligand-directed nanoparticle through systemic administration, a siRNA-mediated anti-angiogenesis activity was able to suppress the symptom of ocular neovascularization induced by HSV infection in mice (13).

When Src homology 2 domain adaptor protein (Shb) was knocked down with Shb-siRNA, VEGF-dependent cellular migration was reduced resulting a loss of stimulation of phosphatidylinositol 3-kinase, phosphorylation of focal adhesion kinase, the generation of focal adhesions, and stress fiber formation in response to VEGF (14). Another protein, IQGAP1, expressed in endothelial cell (EC) was found involving in VEGF-stimulated ROS production, Akt phosphorylation, endothelial migration and proliferation, through siRNA-mediated IQGAP1 knockdown (15). Using neuroblastoma syngenic tumor model (16) and the HSV-induced ocular neovascularization model (13), siRNA duplexes specific to murine VEGFR2 were able to inhibit angiogenesis phenotypes and resulted in significant reductions of the tumor growth and neovasculature areas. Similarly, siRNA duplexes specific to murine VEGFR1 were also able to demonstrate the anti-angiogenesis effects in the mouse ocular model (13).

The above results have indicated that by using the specific siRNA oligos to inhibit the expressions of HIF-1α, Shb, IQGAP1, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-R1 and VEGF-R2 one is able to achieve the anti-angiogenesis effects in mammalian cell cultures and in various animal disease models.

Matrix Metalloproteases and Adhesion Molecules

Although most vascular BM components sustain the growth, survival and health of vascular endothelium, the matrix metalloproteinases (MMPs) and vascular integrins have emerged as key mediators of angiogenic and anti-angiogenic actions. MMP9 and MMP2 have been shown to be important for mobilization of sequestered VEGF and initiation of tumor angiogenesis (17), while integrins are the major mediators for the interactions between endothelial cells and BM components which activate integrin-receptor signaling and cell functions such as proliferation (18).

MMP-9 knockdown with specific siRNA (19) concomitantly resulted in increased levels of surface E-cadherin, redistribution at the plasma membrane of β-catenin, and its physical association with E-cadherin. In a cell culture assay, reduction of bovine aortic smooth muscle cell (BASMC) migration caused by incubation with conditioned media can be completely reversed by siRNA specific knockdown of MMP-2 (not MMP-9) expression (20). To indirectly block the induction of MMP activity, a novel matrix metalloproteinase regulator, RECK, was specifically down regulated using siRNA duplexes (21). The decrease of RECK expression resulted in inhibition of MMP-2

activation. In a different study, siRNA-mediated knockdown of an endogenous and ubiquitous Mint isoform protein, Mint-3, resulted in inhibition of membrane type 5 matrix metalloproteinases (MT5-MMP) activity (22), implicating Mints as the adaptor protein regulating trafficking of MT-MMPs.

Cleavage of CD44, a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration, contributes to the migration and invasion of tumor cells. It can be suppressed by metalloproteinase inhibitor KB-R7785 and tissue inhibitor of metalloproteinases-1 (TIMP-1), but catalyzed by metalloproteinase-disintegrin ADAM10 that was demonstrated with siRNA-mediated knockdown (23). A different cell surface molecule, CD13/aminopeptide N (CD13/APN) has been identified as a potent regulator of angiogenesis and its transcription in endothelial cells is induced by VEGF through RAS/MAPK pathway. The role of Ets-2 in induction of CD13/APN was revealed when Ets-2 mRNA and protein were down regulated using Ets-2 specific siRNA (24). Another metalloprotease disintegrin ADAM12 was characterized by siRNA-mediated knockdown in C2C12 myoblast cells, showing that ADAM12-mediated adhesion and/or signaling plays a key role in determination of the pool of reserve cells during myoblast differentiation (25). This protein has also been recognized as a key enzyme implicated in ectodomain shedding of membrane-anchored proHB-EGF dependent EGFR transactivation (26). When siRNA specific to Pacsin 3, a protein bound to ADAM12, was used in HT1080 cells, the knocking down of this endogenous gene attenuated the shedding of proHB-EGF induced by TPA and angiotensin II. Focal adhesion kinase (FAK) plays critical role in adhesion to collagens. Using FAK specific siRNA, knocked down FAK expression and resulted in significantly greater inhibition of adhesion not only to collagen I but also to collagen IV and fibronectin (27).

Integrins can transduce signals directly to intracellular molecules and also collaborate with other membrane receptor-mediated signal pathways, including the TGFβ1 pathway. When the effects of cell adhesion status on the TGFβ1-mediated Erk1/2 regulation was evaluated in a gastric carcinoma cell variant (28), the role of Smad protein in enhancing TGFβ1-mediated Erk1/2 activation was revealed when a Smad2-siRNA was transfected into the cells. Smad2 protein is a signal transducer and transcriptional modulator regulating multiple cellular processes, such as cell proliferation, apoptosis, and differentiation. Effects of carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) crosslinking on pancreatic adenocarcinoma cellular interaction with

extracellular matrix components, vitronectin (αvβ3 integrin) and fibronectin (α5β1 integrin), was characterized using siRNA-mediated knockdown of CEACAM6 leading to increased ECM component adhesion (29). The integrin-linked kinase (ILK) stimulated the expression of VEGF by stimulating HIF1α protein expression in a PKB/Akt- and mTOR/FRAP-dependent manner. When specific siRNA knocking down ILK expression, significant inhibition of HIF-1α and VEGF expression, as well as VEGF-mediated endothelial cell migration, capillary formation *in vitro*, and angiogenesis *in vivo* were observed (30). These data demonstrated the essential role of ILK in two key aspects of tumor angiogenesis: VEGF expression by tumor cells and VEGF-stimulated blood vessel formation. Interestingly, a widely expressed focal adhesion protein, PINCH-1, was found to form a ternary complex with ILK and α-parvin (31). Using siRNA-mediated knockdown, PINCH-1 and ILK, but not α-parvin, were found to be critical for prompting cell spreading and motility..

The cooperative roles of PDGFr and αvβ3 integrin in the glioblastoma cell migration were investigated by PDGF stimulation of vitronectin-adherent cells, resulting promotion of the specific recruitment of αvβ3 integrin (32). In the vitronectin-adherent cells, Lck/yes-related novel protein (Lyn) was associated preferentially with αvβ3 integrin both in the presence and absence of PDGF stimulation. Down-regulation of Lyn expression using specific siRNA resulted in significant inhibition of the cell migration mediated by αvβ3 integrin in PDGF-stimulated cells. In fibrotic liver, the fate of stellate cells was influenced by extracellular matrix through an intermediary of αvβ3 integrin that was proven using siRNA to silence α(v) subunit (33). In a different study, microfilaments were found to associate with αvβ3 integrin-positive focal contacts in endothelial cells. When vimentin expression was inhibited by siRNA, the cells assembled smaller than normal focal contacts, and showed decreased adhesion to the substratum (34). A laminin adhesion receptor, α6β4 integrin, was studied using siRNA for its role in the invasive phenotype of many carcinomas. The siRNA inhibitors targeting either subunit of the α6β4 integrin were able to reduce cell surface expression of this integrin and to decrease invasion of MDA-MB-231 breast carcinoma cells (35).

The above experimental results demonstrated that using the specific siRNA oligos to inhibit the expressions of various membrane associated factors: αvβ3 integrin, α6β4 integrin, Lyn, PDGFr, ILK, CEACAM6, Smad2, FAK, Pacsin 3, ADAM12, Ets-2,

ADAM10, Mint-3, MMP-2 and MMP-9, will result in down regulation of angiogenic activities in various cell types.

Other Receptors Involved in Angiogenesis

In addition to VEGF and PDGF receptors, down regulation of epidermal growth factor receptor (EGFR or erbB1) presents a promising anti-angiogenesis opportunity. When expression of endogenous erbB1 was knocked down specifically and extensively (90%) in A431 human epidermoid carcinoma cells using RNAi, EGF-induced tyrosine phosphorylation was inhibited and cell proliferation was reduced due to induction of apoptosis (36). On the other hand, using a retrovirus-mediated transfer of Her-2/neu-siRNA, the infected breast and ovarian tumor cells exhibited slower proliferation, increased apoptosis, increased G0/G1 arrest, and decreased tumor growth (37). Knockdown of Her-2/neu expression by siRNA was also associated with increased expression of the antiangiogenic factor thrombospondin-1 and decreased expression of VEGF, indicating that Her-2/neu stimulates tumor growth in part by regulating angiogenesis. When pSUPER plasmid vector expressing siRNA specific to S100A10, a key plasminogen receptor of the extracellular cell surface, was transfected in colorectal (CCL-222) cancer cells, down-regulation of S100A10 resulted in a 45% loss of plasminogen binding and a complete loss in plasminogen-dependent cellular invasiveness (38). Using inducible RNAi mediated knockdown of endogenous CXCR4, a protein playing pleiotropic roles in angiogenesis, host immune response, homing and tumor metastasis, resulted in a significant inhibition of breast cancer cell migration *in vitro* (39).

A pro-apoptotic protein Bim (40) was identified as a critical mediator of anoikis in epithelial cells when knockdown of Bim expression by siRNA resulting inhibited anoikis. To reveal the communication between G protein-coupled receptor (GPCR) and EGF receptor (EGFR) signaling systems involved in cell surface proteolysis of EGF-like precursors, the role of amphiregulin (AR) was evaluated using siRNA inhibition that resulted in prevention of GPCR-induced EGFR tyrosine phosphorylation, downstream mitogenic signalling events, cell proliferation, migration and activation of the survival mediator Akt/PKB. In the same study (41), using siRNA to silence metalloprotease-disintegrin TNFα-converting enzyme (TACE) was able to suppress GPCR-stimulated AR release, EGFR activation and downstream events.

The above experimental results demonstrated that using the specific siRNA oligos to inhibit the expressions of other receptor associated factors: TACE, Amphiregulin, CXCR4, S100A10, Her-2/neu and ErbB-1, will up-regulate apoptotic activities of epithelial cell and block cell proliferation.

Factors in Signal Transduction Pathways

In general, VEGF promotes angiogenesis by stimulating migration, proliferation and organization of endothelium, through the activation of signaling pathways. VEGF stimulation of sphingosine kinase (SPK) affects not only endothelial cell signaling but also tumor cells expressing VEGF receptors (42). In T24 bladder tumor cells, VEGF treatment reduced cellular sphingosine levels while raising that of sphingosine-1-phosphate. SiRNA that targets SPK1, but not SPK2, was able to block VEGF-induced accumulation of Ras-GTP and phospho-ERK, but not EGF induced accumulation of phospho-ERK1/2. To reveal the involvement of diacylglycerol kinase-alpha (Dgkα) in hepatocytes growth factor (HGF)-stimulated cell migration, specific knockdown of Dgkα by siRNA was able to impair angiogenesis *in vitro,* indicating that Dgkα is essential for both proliferative and migratory response to VEGF, and showing it constitutes a novel pharmacological target for angiogenesis control (43). In a different study, inhibition of Mcl-1 by siRNA led to decreased proliferation and induction of apoptosis, supporting the notion that VEGF-induced MM cell proliferation and survival is mediated via Mcl-1, and providing the preclinical framework for novel therapeutics targeting both Mcl-1 and/or VEGF to improve patient outcome in multiple myeloma (44). A growth arrest-specific protein 1 (Gas1) can be up-regulated by junctional membrane protein vascular endothelial cadherin (VEC) in 293 cells. Gas1 siRNA in cell and allantois organ cultures induced endothelial cells refractory to the anti-apoptotic effect of VEGF. Therefore, Gas1 represents another drug target involved in the angiogenesis process (45).

One of the key factors in the downstream signal transduction pathway of VEGF receptors is B-Raf kinase that constitutively activates the MEK/ERK pathway. This increased B-Raf and melanoma cell proliferation, were strongly diminished by siRNA-mediated depletion of the mutant B-Raf protein (46). When the focal adhesion kinase (FAK) was silenced using siRNA in the primary human colon cancer cells and SW620 colonocytes, the pressure-stimulated adhesion was prevented and the pressure-activated FAK397, Src, and FAK576 phosphorylation were also ablated (47). ILKAP, integrin-

linked kinase-associated serine/threonine phosphatase 2C, selectively associates with integrin linked kinase, ILK, to modulate cell adhesion and growth factor signaling. When ILKAP suppressed with specific siRNA, cell entry to the S phase increased, consistent with ILK antagonism (48). The role of diphosphoinositol polyphosphates (DIP) was also characterized by siRNA-mediated down regulation indicating that DIP inactivated Rho and activated Rac following EGF stimulation in the membrane fraction (49). Using siRNA to inhibit Disabled-2 protein (DAB2), expression in K562 cells resulted in modulation of cell-cell adhesion and mitogen-activated protein kinase (MAPK) phosphorylation (50). SiRNA inhibitor knocked down PDK1, 3-phosphoinositide-dependent protein kinase-1, demonstrating that PDK1 is associated with maintaining the steady-state phophorylated MEK level and cell growth (51). Interestingly, the down regulation of PDK1 reduced MEK and MAPK activities but could not prolong MAPK signaling duration. Interaction of TRIP6 (thyroid receptor interacting protein 6)/ZRP-1 (zyxin-related protein 1) to lysophosphatidic acid (LPA) receptor was induced by LPA associated with activation of actin rearrangement, focal adhesion assembly, and cell migration (52). When TRIP6 was silenced by siRNA, its role in LPA-induced cell migration was revealed in SKOV3 ovarian cancer cells.

The above experimental results demonstrated that using the specific siRNA oligos to inhibit the expressions of various signal transduction factors: TRIP6, PDK1, DAB2, ILKAP, B-Raf, Mcl-1, Dgkα and SPK1, will lead to blocking VEGF induced angiogenesis.

Anti-VEGF siRNA to Treat Cancer

There are three distinct approaches to achieve anti-angiogenesis activity for cancer treatments: (A) Activating endogenous anti-angiogenesis factors. (B) Exogenously delivering anti-angiogenesis factors. (C) Delivering inhibitors to reduce activity of endogenous pro-angiogenesis factors. RNAi is a particularly useful means to inhibit activity of drug targets due to its high potency in a sequence specific manner, which has been well-demonstrated in the cell culture studies for anti-angiogenesis activities. However, therapeutic use of RNAi is realized when *in vivo* anti-angiogenesis efficacy of the siRNA agents is achieved with clinically feasible delivery systems (53).

Using intratumoral delivery of anti-VEGF siRNA, inhibition of tumor growth was observed in several xenograft tumor models using human breast carcinoma cells of MDA-

435 and MCF-7 (10). Those siRNA agents with *in vivo* validated activities were further systemically administrated to the mice bearing neuroblastoma tumors with a ligand-directed nanoparticle carrier, resulted in significant tumor inhibition after repeated dosing (16). It has been recognized that local delivery of anti-tumor agents is limited to a few of tumor types with little clinical relevance. Therefore, the systemic administration of siRNA duplexes will be greatly appreciated as a clinically viable delivery for treatment of metastasis cancer. In tumors expressing endogenously both thrombospondin-1 (TSP1) and VEGF, the effects of TSP1 reducing vascularization and tumor growth were recovered when VEGF was down regulated using a systemic administration of crude anti-VEGF siRNA (54). In the same report, surprisingly, when the siRNA was delivered locally into the tumors, no change of VEGF expression was observed. In a different study, an intratumoral delivery system was used along with atelocollagen as a carrier to administrate siRNA targeting human VEGF expressed from PC-3 subcutaneous xenograft tumors, resulting in dramatically suppressed tumor angiogenesis and tumor growth (11). The above preclinical efficacy studies represent a growing effort for siRNA-based anti-angiogenesis therapeutics for treatment of malignancy.

To achieve in vivo anti-angiogenesis efficacy with multiple siRNA inhibitors, various delivery carriers and routes described above are applicable. Intratumoral delivery of siRNA with chemically synthesized reagents, such as liposome, polymer or other aqueous solution, or with 5% glucose and saline buffer, followed by electroporation, ultrasound or other enhancement, are effective means for allowing siRNA inhibitors to be transfected into tumor cells. Using RGD ligand directed nanoparticle containing multiple siRNA inhibitors, a systemic dual-targeted (neovasculature targeted and pro-angiogenesis gene targeted) siRNA delivery approach are clinical viable and can be used for siRNA therapeutics to treat human cancers.

There are other pathways that play very critical roles in tumor growth, e.g. Growth factors, Cytokines, Kinases and Transcription factors. Many of the factors involving the related pathways over-expressed in the tumor tissues, will be good targets for siRNA-mediated knockdown for cancer treatment. Down regulation of multiple cancer causing genes either from the same pathway or different pathway with multiple siRNA inhibitors, achieves much stronger anti-cancer efficacy for the treatment. The current clinical practice for cancer treatment often involves combining different therapeutic approaches, and different drug modalities.

Inflammatory diseases

Rheumatic diseases

Rheumatic diseases, like rheumatoid arthritis, scleroderma, lupus, polymyositis, dermatomyositis, fibromyalgia, psoriatic arthritis, ankylosing spondylitis, Reiter's syndrome, and juvenile rheumatoid arthritis, are the most common autoimmune diseases. They include seriously debilitating diseases that affect over 1% of the population in the developed world, accounting for millions of patients worldwide. The disease is due to a local inflammatory reaction, causing pain and impairing normal organ functioning affecting patients' daily activities.

The key to therapeutic efficacy is delivery to the affected skin area and/or components of the musculoskeletal system; the RNAi agents must act locally at the disease site to be effective. This invention relates to several strategies to achieve delivery to both skin and musculoskeletal system. As proteins are also implicated as possible targets in musculoskeletal diseases (like osteoarthritis, osteoporosis, osteomyelitis, spinal stenosis, heritable disorders of connective tissue, bone formation disorders, Paget's disease, Behcet's disease, bursitis/tendonitis, gout, Duchenne's muscular dystrophy, myotonic dystrophy, limb girdle muscular dystrophies, several diseases of muscle ion channels or contractile proteins and neoplastic diseases) and skin diseases (like vitiligo, psoriasis, alopecia areata, imperfect wound healing, neoplastic diseases, pemphigus, pemphigoid, acne, avascular necrosis, atopic dermatitis, scleroderma, rosacea, heritable disorders of connective tissue, dermal infections and inflammations, pruritus, and hypertrophic scars and keloids) our demonstrated ability to deliver RNAi agents to these tissues and to silence specific proteins implies that this invention can provide relief of disease symptoms for these disease classes as well.

Several treatments exist to suppress the inflammatory episodes that are the hallmark of these diseases. Unfortunately, these agents can lack efficacy or cause severe side effects and tolerance to their therapeutic action can occur. A new treatment for rheumatic diseases that is both potent and avoids side effects or is able to add additional benefit to other treatment options (like corticosteroids, antibodies, antisense, gene therapy, soluble receptors, decoy receptors, receptor (ant)agonists, etc.) would mean a significant health benefit. siRNA inhibitor can be used to knock down over expressed TNF, IL-1

and their receptors in the mammalian cells. By knocking down several of those cytokines and their receptors at the same time, the inflammatory activities are blocked so that the disease progression of the symptoms is alleviated.

Eye disease

Ocular neovascularization (NV), abnormal proliferation of new blood vessels within the eye, is an early pathological step of many eye diseases and is the most common cause of permanent blindness in United States and Europe. There are several major eye diseases promoting the abnormal neovascularization and resulting further damage to the eyes.

Diabetic Retinopathy (DR) occurs when damage to the tiny blood vessels which provide oxygen to the retina become damaged. The damage allows blood and fluid to escape into the retina and can also result in new blood vessel growth. These new vessels are even more fragile and frequently bleed into the vitreous. Patients with the most serious form of DR are at a substantial risk for severe visual loss without treatment. Here, the neovascularization is the result of the disease and it makes matters worse. All are caused by multiple unwanted expressions of certain disease genes.

The predominant symptoms of those eye diseases is ocular neovascularization, which is the major cause of blindness in the patients. The neovascularization process is the result of the over expression of the VEGF proteins and their receptors. Therefore, knocking down these pro-angiogenesis genes is an effective approach, and combining multiple siRNA inhibitors achieves a stronger effect.

A viral infectious disease can be treated either by knocking down viral genes or by modulating expression of cytokine genes of patient.

Infectious Diseases

Many diseases are the result of infection, or exacerbated by infection. Progress has been made in developing treatments for viral infections but even after many years of work on any one virus, inadequate treatment persists. When new viral infections appear, such as the SARS corona virus, new treatments are needed. The invention relates to combination treatments of infectious disease, and disease exacerbated by infection, such as HPV cervical cancer. The methods and compositions described below provide are

applicable in treating infections and combination not described below, or even for viruses not yet known.

Targeting SARS coronavirus for treatment of SARS

Many infectious diseases have claimed human lives throughout the human history. The recent SARS epidemic in China and Canada has killed hundreds of people. Scientists in many laboratories in Asia, Europe and North America have been working diligently on determining the cause of SARS. A previously unrecognized coronavirus in patients with SARS has been isolated, sequenced and tested in a monkey model. This new coronavirus, which is the leading candidate for causing SARS, has been named SARS coronavirus by the World Health Organization. SARS coronavirus a sense and single stranded RNA, can cause one of the most prevalent infections in humans. The virulence of SARS coronavirus results from i) its easy spread by aerosol and other person-to-person contacts, ii) its ability to escape from protective immunity by frequent changes in viral antigens (antigenic drift, like influenza virus), and iii) the sharp emergence of new virulent strains of the virus by reassortment or mixing of RNA segments between viruses from two different species (antigenic shift). The threat of this new strain of SARS coronavirus is so severe because, despite intensive efforts, no effective therapy or vaccine is yet available for prevention and treatment of the SARS coronavirus infection. SARS CoV proprotein replicase 1 (pp1) is the first and only gene product expressed using the viral RNA genome as template. The pp1a and pp1b (Figure 1a) are further processed into approximate one dozen non-structural proteins. The nsp-1 is probably a proteinase important for the maturation of viral proteins. The nsp-9 is a RNA dependent RNA polymerase that catalyzes the synthesis of viral RNAs. We predicted that siRNA sequences targeting nsp-1 and nsp-9 coding regions would be the most effective inhibitors of the SARS CoV RNA transcription and replication. Spike protein, located on the surface of virion, is responsible for tropism, receptor recognition, cell adsorption, and induction of neutralizing antibody. Thus the Spike coding region is very likely to be an effective target for blocking spread of viral infection.

Targeting RSV for treatment of respiratory infection

Infection caused by Respiratory syncytial virus (RSV) is another example of respiratory viral disease for that this present invention will target. RSV infection is the major cause of serious pediatric respiratory tract disease. About two-third of infants are infected with RSV during the first year of life and almost 100% have been infected by age 2. RSV infection tends to occur in sudden outbreaks that last for several months. In fact, to most of viral respiratory infections there is no specific and effective therapeutics available that can substantially reduce the viral burden in patients. For example, even the recently marketed anti-RSV monoclonal antibody, Synagis (Medimmune, Inc), could not reduce the severity of RSV infection among hospitalized patients who received Synagis compared to those who received placebo. This can be at least partly attributable to the uselessness of antibody to neutralize the virus inside the infected cells. The efficacy of antiviral agent ribavirin (ICN Pharma.) has been controversial. In the case of RSV, and other viral infections as well, there are huge market needs for potent and safe modalities that can kill the virus inside cells, effectively inhibiting viral replication, or inhibiting host factors that contribute to the pathogenesis.

According to above description of virus replicative cycle and the viral pathologenesis, the bases for the application of siRNA for treatment of RSV infection are: siRNA could be applied to cells/tissues in early period of viral replication, that is, before or right after viral infection. siRNA degrades the mRNAs synthesized in the "primary transcription" step, instead of waiting till the secondary transcription brings the burst of mRNAs. siRNA are most effective for the degradation of sub-genomic mRNAs instead of genomic RNAs, because the former is not blocked by N protein. It is reasonable to target the ORFs proximal to the 5'- end of the genome. For example, the ORF of L. It is expressed in a smaller amount than other ORFs, and it works on in a catalytic amount; therefore, its silencing requires a lower dose of siRNA. To inhibit viral replication or to protect cells/tissues from viral infection, four key genes (L, F, G, and P) are reasonable to be firstly considered candidate targets for the siRNA-mediated silencing. Although a preliminary study in tissue culture has tested the silencing of proteins F and P, no other genes have been reported, and no more siRNAs targeting same gene have been reported. Since the N protein builds up a protective "firewall" around the genomic RNAs that makes the RNA extremely resistant to RNase, some researchers postulated that it would also block access of siRNA to viral genomic, limiting the possibility of using siRNA to

silence the virus through inhibition of genomic RNAs or cis-acting elements. However, during the transcription/replication processes, the 3'-end N-RNA complex dissociates, and/or the RNAs unwind, exposing the genomic RNA and cis-acting elements to siRNA inhibition.

Multiple-targeting strategies can be used in applying siRNA technology. That means siRNAs targeting different sequences are to be used simultaneously in one application, either *in vitro* or *in vivo*. These targets used in this strategy include two concepts: multiple target sequence of a single gene, and multiple gene targets (viral- or viral plus host genes).

siRNA can be delivered into the "respiratory tree" to treat RSV infection. siRNA could be delivered through nasal spray or inhalation. Since nasopharynx is the major place of virus manifestation in early stage of infection, for prophylactic and early therapeutic treatment, upper respiratory tract delivery of siRNAs will be enough. Deeper delivery of siRNA can be performed, such as when infection spreads to lower regions of the respiratory tissues. In such cases, instillation into trachea, and inhalation of aerosolized solutions can be used. Means to overcome the inflammatory secretions or mucus forming physical barrier to delivery of siRNA can also be used.

Therefore, knocking down multiple viral mRNA strengthens the blockage of the viral protein production, resulting in potent inhibition of the viral infection and replication. It has been demonstrated that the siRNA duplexes targeting these regions indeed inhibited the SARS coronavirus infection and replication in the non-human primate cell culture. In present invention, the combination of multiple active anti-SARS siRNA is more effective than a single siRNA.

RNAi Therapeutics

Use of RNA interference (RNAi) has been developing rapidly in cell culture and model organisms such as Drosophila, C. elegans, and zebrafish. Studies of RNAi have found that long dsRNA is processed by Dicer, a cellular ribonuclease III, to generate duplexes of about 21 nt with 3'-overhangs, called short interfering RNA (siRNA), which mediates sequence-specific mRNA degradation. As RNAi was chosen as the "Breakthrough of the Year 2002" by *Science*, scientists believe that understanding the mechanisms of RNAi and its rapidly expanding application represent a major breakthrough during the last decade in the field of biomedicine.

This invention relates to several modalities of RNAi agents. In one embodiment, the invention provides a combination of siRNA oligonucleotides. In another embodiment, the invention provides DNA or RNA that express active forms of RNAi oligonucleotides. In yet another embodiment, the invention provides a single molecular entity comprised of siRNA operatively linked molecularly, in one instance by nucleic acid, in another instance by chemical conjugates via the "sense" strand, and in yet another instance by hydrophilic polymers. Another modality of RNAi is inhibition of specific genes via control of transcription. One form of RNAi controlling gene transcription involves miRNA oligonucleotides that have a stem-loop hairpin but a base mismatch within the stem. The methods and compositions of RNAi agents are known to one skilled in the art. The present invention comprises the combination of RNAi agents.

siRNA Oligonucleotides

Use of siRNA duplexes to interfere with expression of a specific gene requires knowledge of target accessibility, effective delivery of the siRNA into the target cells, and, for some biological applications, long-term activity of the siRNA in the cell. Along with fast growing literature on siRNA as a functional genomic tool, there is emerging interest in using siRNA in therapeutics. Therapeutic applications require optimized local and systemic delivery methods. The advantages of using siRNA as a therapeutic agent are due to its specificity, stability and mechanism of action. Since each 21 nt double-stranded RNA oligo has its unique sequence specificity, combination of multiple siRNA duplexes down regulates multiple target genes and results in a synergistic effect. Numerous combinations of siRNA target multiple genes, and those combinations inhibit expression of endogenous or exogenous genes or both. In the present invention, the combination of multiple siRNA targeting multiple genes provides potent inhibition or treatment of disease.

RNAi agents based on siRNA can use other oligonucleotide forms, including short hairpin oligonucleotides, long dsRNA, blunt end dsRNA oligonucleotides, and oligonucleotides coupled together or to other moieties. The invention provides for combinations of RNAi agents, and siRNA agents, as recognized by one skilled in the art.

Expressed RNAi Agents

RNAi agents can use expression cassettes to produce the active species for inhibition of the target gene, including plasmid DNA, viral vectors, and mRNA. The expression of RNAi active species produces sequence specific inhibition of the target gene. The combination of expressed RNAi agents according to the invention comprises agents operative if coupled together with one molecular entity or operatively coupled together with multiple molecular entities. The operative coupling can use an internal ribosome entry site (IRES) or it can use multiple promoters or it can use ribozyme sequences also operatively coupled to cleave the expressed RNA into the active RNAi agents.

The combinations according to the invention are not limited to these embodiments, as can be understood by one skilled in the art. Specific embodiments are described below.

1. The siRNA duplexes, or 21 nt double-stranded RNA oligos, used for gene expression knockdown, regardless of the targeted genes, can be synthesized as the same chemical form. Therefore, the mechanism of action of this drug modality is the same or similar, regardless of the effect on the target gene. The combination of multiple siRNA duplexes in one drug dose reduces the risk of unexpected adverse or toxic side effects.

2. According to the invention, using a combination of siRNA targeting different genes controlling the disease pathology, has a better therapeutic effect than using siRNA targeting an individual gene in the disease pathology. A combination also can have an advantage in permitting a reduced dosage, demonstrating additive or synergistic effects of a combination targeting multiple drug target genes.

Ocular neovascularization is the typical pathological symptom for many eye diseases. siRNA targeting VEGF A, VEGF R1 and VEGF R2 genes provided inhibitors to knock down the corresponding genes, all known to play a key role, and thus targets for drugs to block the angiogenesis process. When each siRNA was delivered separately, the induced angiogenesis was significantly inhibited, regardless of delivery with either local or systemic administration. When a combination of the siRNA was used, thus targeting all three genes simultaneously, the inhibition of the angiogenesis pathology was much

more effective, even though the dosage of each siRNA was reduced, so that the total siRNA dose was the same.

Item 3 demonstrates that the combination of multiple siRNA inhibitors efficiently inhibits multiple endogenous genes in the same VEGF pathway. The knockdown of these multiple genes is much more effective and more potent than knockdown of any one pro-angiogenesis factor as exhibited by a reduced pathological angiogenesis induced by the inflammatory HSV DNA.

The siRNA targeting sequences may comprise the regions containing 21 nt within the mRNA sequence of the gene of interest. The targeting sequences are selected through various algorithms designed for siRNA sequence identification first as indicated in Figure 17. Those sequences are then subjected to screening with various biological assays, e.g. gene silencing potency in mammalian cell culture: readout with RT-PCR, or Western Blot, or ELISA, protein function, or physiology function, etc, demonstrated in Example 1 and 2. The most potent siRNA duplexes were selected for further preclinical study and clinical study.

The selected siRNA duplexes specific to each particular gene can be used together or independently in the combination with other siRNA duplexes specific to other genes. It means that in each combination targeting three genes there are at least three siRNA duplexes.

The ratio of each siRNA duplex targeting one particular gene in the combination can be equal, in terms of molecular weight, or can be different when the targeted sequences, mRNAs of the genes of interest, are expressed at different levels.

Due to the sequence specificity issue regarding different testing organisms i.e. sequences of the same gene from different organisms are usually different although sometimes with high homology, finding the siRNA sequence that can target both human sequence and testing animal sequence (Figure 17, SS1, SS2, SS3 and SS4, etc.) is the best way to have the siRNA inhibitors working for both preclinical study and clinical study.

Alternatively, if the potency of the siRNA duplex targeting both human and testing animal sequence is not as good as the siRNA duplex only targeting human sequence, validated in the cell culture study, the choice of siRNA inhibitor for clinical testing will be the one validated in human cell culture and supported by animal model data when the same gene being inhibited with a different siRNA targeting testing animal.

The invention provides for combination of multiple siRNA inhibitors targeting multiple genes and provides for additional steps for siRNA sequence design. The steps in design of RNAi agents according to the invention includes verification of a lack of sequence homology between the selected RNAi sequences in addition to all other criteria in the RNAi design (minimizing off target homology, specificity for the target sequence (s), fit for RISC (RNA induced silencing complex) binding, etc.) The verification can be done with bioinformatic tools or direct comparison between the sequences, according to methods known to the skilled artisan.

Different combinations in the same pathway with different siRNA inhibitors specific to other VEGF pathway factor mRNA sequences will also be effective, such as combinations of siRNA inhibitors targeting:

VEGF-A, VEGF-B and VEGF-R2

VEGF-A, VEGF-B and PIGF

VEGF-B, VEGF-R1 and VEGF-R2

VEGF-A, PIGF and VEGF-R2

or other combinations targeting the above factors.

Different combinations with different siRNA inhibitors specific to mRNA sequences of factors of matrix metalloproteases and adhesion molecules also be effective, such as combinations of siRNA inhibitors targeting:

MMP-2, MMP-9 and PDGF-R

αvβ3 integrin, αvβ5 integrin and α6β4 integrin

Lyn, PDGFr and ILK

CEACAM6, Smad2 and FAK

Pacsin 3, ADAM12 and Ets-2

Mint-3, MMP-2 and MMP-9

or other combinations targeting the above factors.

Different combinations with different siRNA inhibitors specific to mRNA sequences of other factors from different receptor pathways involved in angiogenesis process will also be effective for treatment of cancer, ocular neovascularization diseases, inflammatory diseases, such as combinations of siRNA inhibitors targeting:

EGF, FGF and VEGF

EGF, VEGF and HGF

ErbB-1, Her-2 and VEGF-R2

ErbB-1, VEGF-R2 and FGF-R

TACE, Amphiregulin and CXCR4

S100A10, Her-2/neu and FGF-R

or other combinations targeting the above factors.

Different combinations with different siRNA inhibitors specific to mRNA sequences of other factors from signal transduction pathways involved in angiogenesis process will also be effective for cancer treatment, such as combinations of siRNA inhibitors targeting:

TRIP6, Grb-2 and PDK1

DAB2, ILKAP and B-Raf

C-Raf, Mcl-1 and Dgkα

A-Raf, Grb-2 and SPK1

or other combinations targeting above factors.

Different combinations with different siRNA inhibitors specific to mRNA sequences of other factors from mitochondria associate proteins involved in apoptosis process will also be effective for cancer treatment, such as combinations of siRNA inhibitors targeting:

Bcl-2, BCL2L1 and Trail

Trail, Bcl-2 and Fas

or other combinations targeting above factors.

Certain combinations of siRNA inhibitors targeting pro-angiogenesis factors, pro-proliferation factors and anti-apoptotic factors will be very effective for certain cancer treatment. For example, combinations of siRNA inhibitors targeting VEGF, EGF, and FGF and their receptors, is a potent drug substance for treatment of lung cancer, renal cancer and colon cancer, while combinations of siRNA inhibitors targeting VEGF, Her-2 and mutant p53 is a potent agent for breast cancer treatment.

Certain combinations of siRNA inhibitors targeting specific sequences of mutant pro-oncogenes have highly selective down regulations of particular polymorphism of various cancer causing genes. For example, a combination of siRNA inhibitors targeting specifically to K-ras mutant, ERCC1 mutant and BRCA1 is very effective for breast cancer treatment.

In the case of cervical cancer, both HPV (Human papillomavirus) and the endogenous oncogenes and proto-oncogenes have been identified as the major causes.

Therefore, the siRNA inhibitors used in the combination target HPV (several strains, e.g. E6 and E7 sequences) and p53, at the same time, with the following combination: E6, E7 and p53. Where E6 and E7 genes are sequences from strain HPV16 or HPV18, and p53 is human mutant sequence.

In case of prostate cancer, a combination of siRNA inhibitors targeting antrogen receptor, VEGF R2 and Alpha-methylacyl-CoA racemase (AMACR) is effective treatment.

For oncology application, a combination of siRNA inhibitors targeting multiple (equal to or more than 3) gene sequences is part of complimentary therapeutic regimen. For example, combination of siRNA inhibitors is applied with a monoclonal antibody drug, antibiotic drug (chemotherapy) and other small molecule drug (Gleevec), etc., for cancer treatment.

Different combinations with different siRNA inhibitors specific to mRNA sequences of other factors involved in inflammation diseases rather than angiogenesis also is effective treatment for those diseases, such as combinations of siRNA inhibitors targeting:

TNF-α, TNF-β and IL-1

TNF-α, IL-1 and IL-1r

IL-1β, GG2-1 and TNF-α

CIAS1, Ark and TNF-α

TNF-β, IL-1α and IL-1 β

NF-κB, TNF- α and IL-1

or other combinations targeting above the factors.

Ocular stromal keratitis is human herpes simplex virus (HSV) infection induced inflammatory disease. A combination of siRNA inhibitors targeting HSV sequence, pro-inflammatory cytokines, e.g., IL-17, IL-12, and angiogenesis factors, VEGF or VEGF R2 is an effective therapeutic.

When SARS virus infection and replication was inhibited in the fetal rhesus kidney cells (FRhK-4), 4 siRNA duplexes, SC2, SC5, SC14 and SC15, targeting respectively the non specified proteins (nsp-1, nsp-9 and nsp-10) and Spike protein showed strong prophylactic effects to viral infection (cells first being transfected with siRNA and then infected with the virus), but relatively weaker effects on the therapeutic effects (cells first infected with the virus and then transfected with siRNA). When

various active siRNA duplexes at different ratios were combined, the therapeutic effects were significantly improved.

Inhibition of RSV infection using combination of siRNA inhibitors targeting viral nucleocapsid protein (N), nonglycosylated inner virion protei (M) and a transmembrane glycoprotein (F) gene sequences through airway administration is an effective approach.

The concept of using the siRNA oligo cocktail (siRNA-OC), multiple siRNA duplexes targeting multiple genes, to down regulation the expression of the disease causing or disease control genes. The combination of the siRNA oligos will have at least even or better effects on the targeted diseases.

The proportion of each siRNA component can be different depending the needs for effective down regulation of the targeted genes and disease status.The invention indicates that the siRNA-OC formulation contains at least 3 siRNA duplexes, the number of the siRNA duplexes can be more, from 4 to 5, 6, 7, 8, 9, 10, or more.

The targeted disease causing genes can be endogenously expressed genes or genes from the infective bacteria, virus and protozoa, etc. The chemical form of siRNA duplexes can be same or different. The siRNA-OC can be delivered either locally or systemically.

The siRNA-OC can be used for either prophylactic or therapeutic effects, or both. The siRNA-OC can be used for treatment of cancer, autoimmune and inflammatory diseases. The siRNA-OC can be delivered in Saline solution or other solutions: liposome, polymer and nanoparticles. The siRNA-OC can be a mixture in powder form. The siRNA-OC also can be combined with other drug substances.

EXAMPLES

The following examples further illustrate the invention and are not to be construed as limiting the invention.

Example 1.

Combination of siRNA duplexes targeting VEGF pathway genes for treatment of ocular neovascularization.

1) Recently, we demonstrated that HSV DNA that contains abundant bioactive CpG-containing motifs can induce the potent angiogenesis factor vascular endothelial growth factor (VEGF) and that neutralization of VEGF with antibody minimized HSV-induced angiogenesis. A convenient model was also established in which bioactive CpGcontaining oligodeoxynucleotides (ODNs) were also shown to induce neovascularization via the induction of VEGF. This model is used to demonstrate the therapeutic value of RNA interference (RNAi) to suppress VEGF expression and responsiveness.

2) Material and Methods

Reagents

Phosphorothioate ODNs were kindly provided by Dennis M. Klinman (Biologics Evaluation and Research, Food and Drug Administration, Washington, DC). The sequences of stimulatory ODNs used in this study were: 1466, TCAACGTTGA, and 1555, GCTAGACGTTAGCGT. Subsequent studies were performed using an equimolar mixture of ODNs 1466 and 1555. *Molecular Design of Gene Targets and siRNAs* Three mVEGF pathway factors, mVEGF A and two mVEGF receptors (mVEGFR1 and mVEGFR2), were targeted by RNAi. For each gene target, two target sequences were assigned at different locations on the same mRNA. siRNAs were designed correspondent to the above target sequences. These siRNAs were designed according to the guideline proposed by Tuschl. (14, 15) The designed siRNAs (duplexes of sense and anti-sense strands) were synthesized by Qiagen (Valencia,CA). All siRNAs were 21-nucleotides long doublestranded RNA oligos with a two-nucleotide (TT) overhang at the 3 prime end. The targeted sequences of mVEGFA were (a) AAGCCGTCCTGTGTGCCGCTG and (b) AACGATGAAGCCCTGGAGTGC.

The targeted sequences of mVEGFR1 were (a) AAGTTAAAAGTGCCTGAACTG and (b) AAGCAGGCCAGACTCTCTTTC. The targeted sequences of mVEGFR2 were (a)

AAGCTCAGCACACAGAAAGAC and (b) ATGCGGCGGTGGTGACAGTA. The synthesis of unrelated siRNA controls, two target sequences each for LacZ and firefly luciferase were used. They were LacZ (a) AACAGTTGCGCAGCCTGAATG and (b) AACTTAATCGCCTTGCAGCAC, Luc (a) AAGCTATGAAACGATATGGGC and (b) AACCGCTGGAGAGCAACTGCA.

Subsequent studies were conducted using an equimolar mixture of a and b for individual siRNA.

Mice

Female BALB/c mice (H-2d), 5 to 6 weeks old, were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed conventionally. All investigations followed guidelines of the Committee on the Care of Laboratory Animals Resources, Commission of Life Sciences, National Research Council. The animal facilities of the University of Tennessee (Knoxville, TN) are fully accredited by the American Association of Laboratory Animal Care.

Virus

HSV-1 strain RE (kindly provided by Dr. Robert Lausch, University of Alabama, Mobile, AL) was used in all of the procedures. Virus was grown in Vero cell monolayers (catalog no. CCL81; American Type Culture Collection, Manassas, VA), titrated, and stored in aliquots at -80°C until used. In Vitro *Efficacy of siRNA*

To test the efficacy of RNAi *in vitro*, the following cell lines were used. RAW264.7 gamma NO (-) cells, ATCC, CRL-2278, a mouse macrophage cell line, expressing endogenous mVEGF-A, and SVR, ATCC CRL-2280, a mouse endothelial cell line bearing receptors for mVEGF. The cells were plated in a six-well plate in RPMI with 10% fetal bovine serum overnight at 37°C in 5% CO2. One day after cell plating, the cells were transfected with different concentrations of siVEGFA or siLuc (at 0, 0.1, 0.5, 1.0, or 2.0 ug/2 ml/well, respectively) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours later RNA from these cells was extracted for reverse transcriptase-polymerase RNA Extraction and RT-PCR). SVR cells (CRL-2280, ATCC) were used to test the efficiency of siVEGFR1-specific knockdown of VEGFR1 gene that is constitutively expressed on these cells. The cells were plated in a six-well plate in Dulbecco's modified Eagle's medium with 5% fetal bovine serum overnight at 37°C in 5% CO2. One day after cell plating, the cells were transfected with different concentrations of siVEGFR1 or siLuc (at 0, 0.1, 0.5, or 1.0 ug/2 ml/well,

respectively) using Lipofectamine 2000. Forty-eight hours later RNA from these cells was extracted for RSPCR to detect VEGFR1 (see RNA Extraction and NATemplate-Specific PCR) (RS-PCR). The 293 cells (CRL-1573, ATCC) were used to transfect with mVEGFR2-expressing plasmid for the detection of knockdown of exogenous mVEGFR2. The cells were plated in a six-well plate in Dulbecco's modified Eagle's medium with 5% fetal bovine serum overnight at 37°C in 5% CO2. One day after cell plating, the cells were cotransfected with plasmid pCI-VEGFR2 (0.2 ug/2 ml/well) and siVEGFR2 (a, b, a + b), or siLuc (0, 0.1, 0.5, or 1.0 ug/well, respectively) using Lipofectamine 2000. Fortyeight hours later RNA from these cells was extracted for RS-PCR to detect VEGFR2.

Corneal Micropocket Assay

The corneal micropocket assay used in this study observed the general protocol of Kenyon and colleagues. Pellets for insertion into the cornea were made by combining known amounts of CpG ODNs, sucralfate (10 mg, Bulch Meditec, Vaerlose, Denmark), and hydron polymer in ethanol (120 mg/1 ml ethanol; Interferon Sciences, New Brunswick, NJ), and applying the mixture to a 15 mm2 piece of synthetic mesh (Sefar America, Inc., Kansas City, MO). The mixture was allowed to air dry and fibers of the mesh were pulled apart, yielding pellets containing 1 ug of CpG ODNs. The micropocket was made ~1 mm from the limbus under a stereomicroscope (Leica Microsytems, Wetzlar, Germany) (four eyes per group) and pellets containing CpG ODNs were inserted into the micropocket. Angiogenesis was evaluated at days 4 and 7 after pellet implantation by using calipers (Biomedical Research Instruments, Rockville, MD) with a stereomicroscope. The length of the neovessels originated from the limbal vessel ring toward the center of the cornea and the width of the neovessels presented in clock hours were measured.4 Each clock hours is equal to 30° at the circumstance. The angiogenic area was calculated according to the formula for an ellipse. A = [(clock hours) x 0.4 (vessel length in mm) x pi] / 2.

In Vivo Delivery of siRNA

For local delivery, siRNA (10 ug/10ul per eye) was diluted in phosphate-buffered saline (PBS) and delivered subconjunctivally. The subconjunctival injections were given by a 32-gauge Hamilton syringe (Hamilton Co., Reno, NV) at 6 and 24 hours after CpG pellet implantation or days 1 and 3 after virus infection under deep anesthesia induced by Avertin (Pittman Moore, Mondelein, IL). siRNA was injected 2 mm behind

the limbus. For systemic injection, siRNA (40 ug/100 ul per mice) was mixed with polymer (TargeTran) and delivered intravenously. The tail vein injections were given at 6 and 24 hours after CpG pellet implantation or days 1 and 3 after virus infection using a 32-gauge syringe.

Corneal HSV-1 Infection

Corneal infections of all mouse groups were conducted under deep anesthesia induced by Avertin, St. Louis, MO. The mice were scarified lightly on their corneas with a 30-gauge needle, and a 2-ul drop containing 1 x 10 exp 5 plaque-forming units (PFUs) of HSV-1 RE was applied to the eye and gently massaged with the eyelids (six mice per group).

Clinical Observations (HSK Severity and Angiogenic Scoring)

The eyes were examined on different days after infection for the development of clinical lesions by slit-lamp biomicroscopy (Kawa Company, Nagoya, Japan), and the clinical severity of keratitis of individually scored mice was recorded. The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing SK. The severity of angiogenesis was recorded as described previously.4 Briefly, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye were then summed to derive the neovascularization index (range, 0 to 16) for each eye at a given time point.4

Statistical Analysis

Significant differences between groups were evaluated by using the Student's *t*-test. $P < 0.05$ was regarded as significant difference between two groups.

3) Results

Knockdown of VEGF-pathway genes by siRNA in vitro.

Three individual siRNAs were developed. These were siVEGFA, siVEGFR1, and siVEGFR2. Each was tested in a different cell system *in vitro* to measure its gene silencing efficiency. The siVEGFA was tested in RAW NO (-) macrophage cells, that produce VEGFA endogenously 18. Cells were transfected with siVEGFA, or control siLuc siRNA at various doses, and RT-PCR was performed 24 h after transfection. As shown is Fig. 1*A*, expression of both the 120 and 164 isoforms of VEGF was reduced

by siVEGFA, in a dose dependent manner. The efficacy of the siVEGFR1 reagent was evaluated in SVR cells that endogenously express the VEGF receptor 1. As shown in Fig. 1*B*, VEGFR1 expression measured by RS-PCR 48 h post transfection was diminished in a dose dependent manner whereas all concentrations of the control siLuc siRNA resulted in similar levels of VEGFR1 signal. Finally the siVEGFR2 reagent was tested in 293 cells that were exogenously transfected with a plasmid encoding VEGFR2. To measure the silencing effect of siVEGFR2, a cotransfection approach using siVEGFR2 and VEGFR2 DNA at various doses was performed. 293 cells were cotransfected with various concentrations of siRNA targeting mVEGFR2 (0.1, 0.5, 1.0 ug/well) and plasmid expressing mVEGFR2 (0.2 ug/well). RNA was isolated 48 h postcotransfection and exogenous expression of mVEGFR2 was measured by RS-PCR. Fig. 1*C* shows that VEGFR2 expression was reduced by siVEGFR2, but not by the control siRNA molecule. These results indicate that all the tested siRNAs were able to suppress the targeted genes *in vitro*.

Inhibition of CpG-induced angiogenesis by local delivery of siRNAs targeting VEGF-pathway genes.

A previous study demonstrated that CpG containing ODN encapsulated in hydron pellets induce VEGF mediated angiogenesis when inserted into corneal micropockets. This system was used to measure the inhibitory effect of local administration of siRNA preparations designed to target VEGF as well as two of its receptors (VEGFR1 and 2). A single dose of 10 ug siRNA in PBS was used in all cases. This was administered by subconjunctival injection 24 h after the establishment of micropockets containing CpG ODN. The siRNAs were tested individually as well as a 1:1:1 mixture of all three (siVEGFA, siVEGFR1 and siVEGFR2). New blood vessel formation in the corneal limbus was monitored at both day 4 and 7 after pellet implantation. As shown in Fig. 2, significant inhibition of corneal neovascularization resulted with all three test siRNAs compared to those given control siLacZ at day 4 after pellet implantation ($p < 0.05$). The combination of the three tested siRNAs was the most effective inhibitor, providing an approximately 60% reduction in neovascularization ($p < 0.01$).

Inhibition of CpG-induced neovascularization by systemic delivery of siRNAs targeting VEGF-pathway genes.

To test the anti-angiogenic effect of targeted individual siRNA and the efficiency of

systemic siRNA delivery, mice with CpG ODN-containing micropockets were given a single dose i.v. of 40 ug siRNAs containing either siVEGFA, siVEGFR1, siVEGFR2, a mix of the three, or control siLacZ 6 and 24 h post pellet implantation. In these experiments a polymer ("Targetran") was used that was shown in previous studies on tumor angiogenesis to facilitate extravascular delivery of siRNA. At day 4 and 7 after pellet implantation, the extent of angiogenesis was measured. As shown in Fig. 3, all reagents used individually induced significant inhibition of neovascularization compared to the siLacZ treated group at day 4 after pellet implantation ($p < 0.05$). As observed with local administration, the mix of the three test reagents provided the most effective inhibition (average 40% inhibition, $p < 0.01$). In additional experiments, the function of the polymer vehicle was evaluated by comparing the anti-neovascularization activity of the test mix suspended in polymer or given in PBS. These experiments revealed that the use of the polymer vehicle resulted in more effective anti16 neovascularization than was evident when the PBS vehicle was used although result was only significant at the early test period ($p < 0.05$) (Fig. 4*A*). The results demonstrate that ocular neovascularization can be controlled by the i.v. administration of siRNA that target the VEGF system genes and that the use of the "TargeTran" vehicle enhanced the efficacy of the therapeutic effect.

To determine the efficient anti-angiogenic dose of siRNA in systemic delivery, mice with CpG ODN-containing micropockets were given a single dose i.v. of 10, 20, 40, 80 ug siRNAs containing a mix of the siVEGFA, siVEGFR1 and siVEGFR2, or control siLuc with TargeTran vehicle at 6 and 24 h post pellet implantation. As shown in Fig 4*B*, administration of siRNAs inhibited CpG induced angiogenesis in a dose dependent manner.

Therapeutic application of siRNAs against VEGF-pathway genes in the HSK model.

Previous studies have shown that VEGF is the critical angiogenic factor for induction of HSV specific angiogenesis in the HSK model. To evaluate whether administration of siRNAs targeting VEGF-pathway genes inhibits the development of HSK, the corneas of mice were scarified and infected with 1· 105 HSV-1 RE. Then mice were given a single dose of 10 ug (subconjunctival injection for local delivery) or 40 ug (tail vein injection for systemic delivery) mix of siRNAs (an equimolar mixture of siVEGFA, siVEGFR1, and siVEGFR2) with polymer vehicle at day 1 and 3 after virus infection. As shown in Fig. 5, the angiogenesis and severity of HSK was significantly

reduced in mice treated with siRNAs targeting VEGF-pathway genes either locally or systemically compared to animals treated with siLuc control ($p < 0.05$). Whilst 80% of siLuc control treated eyes developed clinically evident lesions (score 2 or greater at day 10 p.i.), only 42% (local delivery) or 50% (systemic delivery) of eyes treated with siRNAs targeting VEGF-pathway genes developed such lesions. In addition by day 10 p.i., the angiogenesis score was greater than 6 in 9 of 12 control eyes, but only in 5 of 12 eyes of mice treated with siRNAs against VEGF-pathway genes by either local or systemic delivery. Taken together these results show that administration of siRNAs against VEGF-pathway genes reduced development of HSK via inhibition of angiogenesis.

Decreased level of VEGF mRNA following treatment of siRNAs targeting VEGFpathway genes in HSV-1 infected cornea.

To address whether treatment of siRNAs against VEGF-pathway genes reduces the level of VEGF mRNA, corneas were collected at day 4 or 7 p.i. from mice that were infected with 1·105 pfu HSV-1 RE and were treated with siRNAs targeting VEGF pathway genes at day 1 and 3 after viral infection. The VEGF mRNA level was measured by RT-PCR or quantitative real-time PCR. As shown in Fig. 6*A*, the expression of VEGF mRNA was reduced in the cornea treated with siRNAs against VEGF-pathway genes compared to control eye at day 4 and 7 post infection. In addition, similar to what is found in RT-PCR, cornea treated with siRNAs against VEGF pathway genes showed the significant reduction in VEGF gene expression in comparison to cornea treated with siLuc control at 7 day p.i.(Fig. 6*B*).

Decreased VEGF protein levels following application of siRNAs against VEGFpathway genes in HSV-1 infected cornea.

To evaluate whether treatment of siRNAs targeting VEGF-pathway genes diminishes the production of VEGF protein, we measured VEGF protein in HSV-1 infected and siRNAs treated cornea at day 7 after infection. As shown in Fig.7, VEGF protein levels were lower in those that received siRNAs targeting VEGF-pathway genes compared to controls given siLuc with polymer ($p < 0.05$). Once again administration of siRNA targeting VEGF-pathway with polymer inhibited the production of its target gene in the HSK cornea.

Example 2

Combination of siRNA duplexes targeting SARS coronavirus sequences for treatment of SARS

1) Prophylactic effect of multiple siRNA combination for inhibition of SARS coronavirus infection and replication in the fetal rhesus kidney cells (FRhK-4). Figure 1. Prophylactic effects of combined siRNA duplexes specific to SARS CoV. A strategy of using combination of active siRNA duplexes to achieve stronger inhibition of viral replication was tested. FRhK-4 cells were transfected and infected as described in Figure 2. At 36 hours post viral infection, the cells and culture medium were collected for QRT-PCR and measurement of $TCID_{50}$. A. Combination of the active siRNA duplexes reduced the viral genome copy. The inhibition effects of the combined siRNA duplexes were measured with real-time Q-RT-PCR and resulted stronger inhibition than that from the single siRNA. B. A time course study using the combined SC2 and SC5 siRNA. The prophylactic effect of combined siRNA against SARS virus was well maintained up to 72 hours post transfection. Number of the combination groups: SC2 + SC5 + SC14; SC14 + SC15; SC14 + SC5; SC14 + SC2; SC5 + SC14 + SC15; 3xSC2: 0.9 μg of SC2 siRNA/well; 3XSC5: 0.9 μg of SC5 siRNA/well and Control: negative control without siRNA transfection.

2) Therapeutic effect of the combined siRNA duplexes on the SARS coronavirus infection and replication in the fetal rhesus kidney cells (FRhK-4). Figure 2. Therapeutic effects of combined siRNA duplexes specific to SARS CoV. Combinations of the active siRNA duplexes were measured. FRhK-4 cells were infected with 3 PFU/cell of SARS CoV followed by transfection with various combination of siRNA duplexes one hour p.i. At 36 hours post transfection, cells and culture medium were collected for Q-RT-PCR and measurement of viral titer, respectively. A. Combined siRNA duplexes were able to improve the inhibition effect significantly ($P<0.05$) measured by reduction of the viral genome copies in the cytoplasm of infected FRhK-4 cells. SC2 + SC5; SC14 + SC15; SC14 + SC5; SC14 + SC2; SC5 + SC15; SC2 + SC5 + SC14 + SC15; SC2 + SC5 + SC14; 2XSC2: 0.6 μg of SC2/well; 2XSC5: 0.6 μg of SC5 siRNA/well; 3XSC5: 0.9 μg of SC5 siRNA/well; 4XSC5: 1.2 μg of siRNA/well and Control: negative control without siRNA transfection.

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